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Proliferation, differentiation, and long-term culture of primary hippocampal neurons

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ABSTRACT **Primary embryonic hippocampal neurons can develop morphologically and functionally in culture but do not survive more than a few weeks. It has been reported that basic fibroblast growth factor (bFGF) promotes the survival of and neurite elongation from fetal hippocampal neurons. We report that bFGF, in a dose-dependent manner, can induce the survival (50 pg to 1 ng/ml) and proliferation (10–20 ng/ml) of embryonic hippocampal progenitor neurons *in vitro*. In serum-free medium containing high concentrations of bFGF, neurons not only proliferated (4-day doubling time) and differentiated morphologically but also could be passaged and grown as continuous cell lines. The neuronal nature of the proliferating cells was positively established by immunostaining with several different neuron-specific markers and by detailed ultrastructural analyses. The proliferative effect of bFGF was used to generate nearly pure neuronal cell cultures that can be passaged, frozen, thawed, and cultured again. Neurons have been maintained >5 months in culture. The ability to establish long-term primary neuronal cultures offers the possibility that clonal lines of distinct neuronal cell types may be isolated from specific areas of the central nervous system. Such long-term neuronal cultures should prove valuable in studying neurons at the individual cell level and also in exploring interactions between neurons *in vitro*. The observed dose dependence raises the possibility that cell survival and proliferation *in vivo* may be influenced by different levels of bFGF.**

During embryonic development neurons and glia are generated from the neuroepithelial cells of the neural tube. Although the factors that control the growth and differentiation of these cells are largely unknown, the survival and growth of central nervous system neurons appear to be regulated by trophic factors. Nerve growth factor, the prototypical neurotrophic factor, supports the survival of cholinergic neurons of septum, striatum, and nucleus basalis (1–3). Basic fibroblast growth factor (bFGF) has a much broader range of effects and supports the survival of a variety of neurons from different regions of the brain (4–8); its most significant effect, however, is on fetal rat hippocampal neurons (4). Both neuronal survival and neurite elongation are increased in the presence of bFGF (4). Moreover, at concentrations >5 ng/ml, bFGF has short-term proliferative and morphodifferentiation effects on neuronal precursor cells from mesencephalon, telencephalon, cerebral hemispheres, and spinal cord (9–11). Proliferation and differentiation of primary neurons from both fetal and adult striatum in response to a combination of nerve growth factor and bFGF, only epidermal growth factor, or bFGF have been reported (12–14). Furthermore, long-term mixed cultures of neurons and glia have been established from fetal and neonatal rat and mouse brains (15, 16). However, in many cases these cell lines were transformed *in vitro* and caused tumors when injected into syngeneic animals (15, 16).

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Unavailability of long-term primary neuronal cultures has made it difficult to address fundamental questions of cellular and molecular interactions among the many functionally distinct neuronal cell types that contribute to the development and functioning of the mammalian central nervous system. Because of the postmitotic nature of neurons and the diversity of cell types in the brain, the long-term culturing of neurons has been a major challenge.

We report that rat embryonic hippocampal neurons respond to bFGF in a dose-dependent manner. Cells proliferate at high concentrations (≥10 ng/ml) of bFGF and survive at lower concentrations. Ultrastructural analysis and immunostaining with antibodies against neuron-specific proteins positively identified the proliferating cells as neurons. We have established nearly pure long-term cultures of neuronal cells that could be passaged, frozen/thawed, and recultured.

MATERIALS AND METHODS

Materials. The materials were from the following sources: Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, N2 supplement, and laminin (GIBCO/BRL); polyornithine (Sigma); recombinant human bFGF (a gift from Syntex/Synergen Consortium, Boulder, CO); bovine bFGF (R&D Systems, Minneapolis); NeuroTag green (Boehringer Mannheim); cell proliferation kit containing bromodeoxyuridine (BrdUrd), anti-BrdUrd antibody and streptavidine/Texas red (Amersham). The antibodies were from the following sources and were used at the indicated dilutions: polyclonal anti-neurofilament 200 (NF; 1:500; Chemicon), monoclonal anti-neuron-specific enolase (NSE; 1:50; DAKO, Carpenteria, CA), monoclonal anti-glia fibrillary acidic protein (GFAP; 1:500–1:10,000; Amersham), monoclonal anti-vimentin (1:800; Boehringer Mannheim), monoclonal anti-OX-42 (1:5000; Serotec), polyclonal anti-galactocerebroside (1:5000; Advanced Immunochemical Services, Long Beach, CA), monoclonal anti-microtubule-associated protein 2 (1:500; Sigma), polyclonal anti-fibronectin (1:2000; Telios, San Diego). Polyclonal nestin antibody (1:15,000) was a gift from R. McKay (Massachusetts Institute of Technology). High-affinity bFGF receptor monoclonal antibody (1:20) was a gift from A. Baird (Whittier Institute, La Jolla, CA). Polyclonal anti-GFAP (1:2000) was a gift from L. F. Eng, (Stanford University).

Cell Culture. Brains of Fischer 344 rats [embryonic day 16 (E16)] were dissected, the meninges were removed, and the hippocampi were isolated. Hippocampi were transferred to 15-ml tissue culture tubes and the volume was adjusted to 1–2 ml with phosphate-buffered saline (pH 7.4) supplemented with 0.6% glucose (PBS-G). Hippocampi were mechanically dissociated by tituration with a Pasteur pipet (≈20 times) followed by tituration (≈20 times) with a reduced-bore Pas-

Abbreviations: bFGF, basic fibroblast growth factor; NF, neurofilament; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; TEM, transmission electron microscopy; SEM, scanning electron microscopy; E, embryonic day.

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teur pipet. The cells were pelleted by centrifugation at 1000 × *g* for 5 min. Cells were taken up in ≈20 ml of N2 medium (17, 18) [1:1 mixture of DMEM/F-12 containing 20 nM progesterone, 30 nM sodium selenite, 100 μM putrescine, 3.9 mM glutamine, insulin (5 μg/ml), and transferrin (100 μg/ml)] and counted in a hemocytometer. Tissue culture plates were coated with polyornithine (10 μg/ml) followed by laminin (10 μg/ml). Approximately 0.5–1.0 × 10^6 cells per well were plated on polyornithine/laminin-coated 6-well plates in N2 medium containing bFGF at 20 ng/ml (N2/bFGF) and cultured at 37°C in 5% CO_2/95% air. Medium was changed every 3–4 days. For passaging, cells were trypsinized (ATV trypsin; Irvine Scientific), taken up in N2/bFGF, and pelleted by centrifugation, and the supernatant was removed. Cells were resuspended in N2/bFGF and plated. Cells could be frozen in liquid nitrogen in N2/bFGF/10% (vol/vol) dimethyl sulfoxide (DMSO). For culturing, cells were thawed quickly at 37°C, added to N2/bFGF, centrifuged to remove DMSO, resuspended in fresh N2/bFGF, and plated.

Dose Dependence. Cells (passage 3) were plated in polyornithine/laminin-coated 24-well plates in N2 containing no bFGF or bFGF at 50 pg/ml to 20 ng/ml. Cells in five randomly chosen areas in each well were counted on days 1, 4, and 7.

BrdUrd Incorporation. Neurons (passage 3) were grown for 3 days, the medium was changed, and the next day the cells were incubated with BrdUrd for 1 or 4 days. Cells were fixed, washed, and treated with a monoclonal antibody against BrdUrd for 1 hr. Cells were washed and incubated with biotinylated anti-mouse antibody (Vector Laboratories) followed by streptavidin–Texas red complex. Stained cultures were examined with a Bio-Rad MRC600 confocal microscope. Epifluorescent and Nomarski transmitted collected images were merged using ADOBE PHOTOSHOP.

Neurotag Binding. Primary neurons (passage 3) grown in culture for 6 days were incubated with recombinant tetanus toxin C fragment conjugated to fluorescein isothiocyanate (NeuroTag; 10 μg/ml) in N2/bFGF and bovine serum albumin (0.1 mg/ml) for 2 h. After washing, the cells were examined in a confocal microscope.

Immunocytochemistry. Cells were passaged (passage 3; 4 days in culture after plating), grown in a 24-well plate, fixed in 4% (wt/vol) paraformaldehyde in PBS, and permeabilized with 0.25% Triton X-100 in Tris-buffered saline. Cells were incubated overnight at 4°C with polyclonal or monoclonal antibodies and 1% normal horse serum (for monoclonal antibody) or 10% (vol/vol) normal goat serum (for polyclonal antibody). After washing, cells were incubated at room temperature with biotin-conjugated goat anti-rabbit IgG or horse anti-mouse IgG antibodies (Vector Laboratories) for 1 h followed by incubation for 1 h with a preformed mixture of avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit). The reaction products were visualized with diaminobenzidine histochemistry.

Electron Microscopy. Cultures (passage 3; 4 days after plating) grown on LabTek slides (Ted Pella, Redding, CA) were fixed in 2% (vol/vol) glutaraldehyde in 100 mM sodium phosphate at 37°C for 2 h, rinsed, and postfixed in 1% aqueous OsO_4 for 1 h at room temperature. They were then dehydrated in a graded ethanol series, infiltrated with Araldite resin, and polymerized *in situ*. The slide was separated from the polymerized resin from which blocks of cells were cut and glued to resin blanks. Sections were cut parallel to the culture substrate at a thickness of 70 nm. Sections collected on 300-mesh copper grids were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM) with a Phillips CM10 TEM at 80 kV. For scanning electron microscopy (SEM), cultures (passage 2; 4 days after plating) grown on LabTek slides were prepared as for TEM through ethanol dehydration. The plastic chambers were then removed, leaving the sealing gasket in place, and the slide was placed in a Pelco critical point dryer. After drying, the slide was critical-point-dried, sputter-coated, and examined in a Cambridge 360 SEM at 10 kV.

RESULTS

The effects of different concentrations of bFGF on neuronal growth were examined (Fig. 1). In the absence of bFGF, cells started to die within 4 days and no cell survived at day 7. bFGF enhanced cell survival; on day 7, 48% and 76% cells had survived in the presence of bFGF at 50 pg/ml and 1 ng/ml, respectively. Similar survival effects of bFGF on hippocampal neurons have been reported (4, 5, 19). bFGF at 10 ng/ml showed a proliferative effect which was more pronounced at 20 ng/ml. However, bFGF at 50 or 100 ng/ml decreased cell proliferation (data not shown). Thus, bFGF has a dose-dependent effect on cell survival and proliferation.

Cells cultured in bFGF at 20 ng/ml began proliferating by day 2 with a doubling time of 4 days (data not shown). Primary cells became contact inhibited for growth and reached a plateau after day 7, although growth continued within aggregates (see Fig. 3*C*). Whether all cells or only a subpopulation were dividing was examined by BrdUrd incorporation. After 1 day, the nuclei of only a small fraction of cells were stained (Fig. 2*A*) but almost the entire cell population was stained after 4 days (Fig. 2*B*).

To establish long-term cultures, cells were trypsinized and passaged. The passaged cells (up to six passages were tested) grew as the original culture. Cells could be frozen in liquid nitrogen, thawed, and recultured. When cells at different passage numbers were thawed and recultured, they grew equally well. Frozen/thawed cells showed the same morphology as those kept continuously in culture (data not shown).

Several independent criteria were used to show that the cultured cells were neurons: morphological characterization, expression of neuronal markers, and ultrastructural analysis. Cell morphology (Fig. 3 *A–C*) was similar to that of short-term neuronal cultures (20, 21). Newborn cells were small and bipolar. By day 2, short processes roughly equal in length to cell bodies were extended from parent cells. In the next 2–3 days, one or two of the processes started to grow rapidly and contacted the neighboring cells (Fig. 3*A*). By day 7, both the cell bodies and the processes had increased in size and an extensive interconnecting network of processes had formed. This morphological progression resembled hippocampal pyramidal neuronal morphologies *in vitro* (20, 21). When cells growing for 1–2 weeks were passaged, more of them had

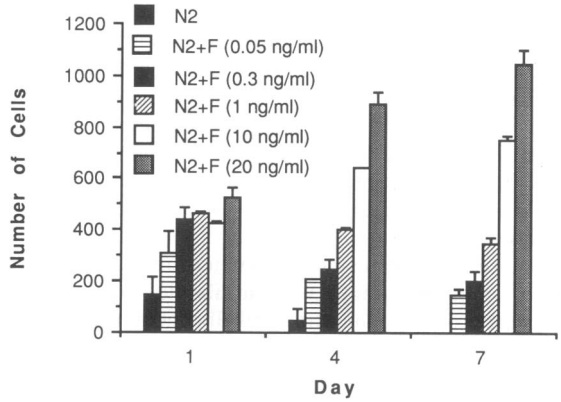

FIG. 1. Dependence of hippocampal neurons on bFGF for survival and proliferation. Cells (passage 3, grown with bFGF at 20 ng/ml until passage) were plated in N2 only or N2 containing various concentrations of bFGF (F) and counted at days 1, 4, and 7.

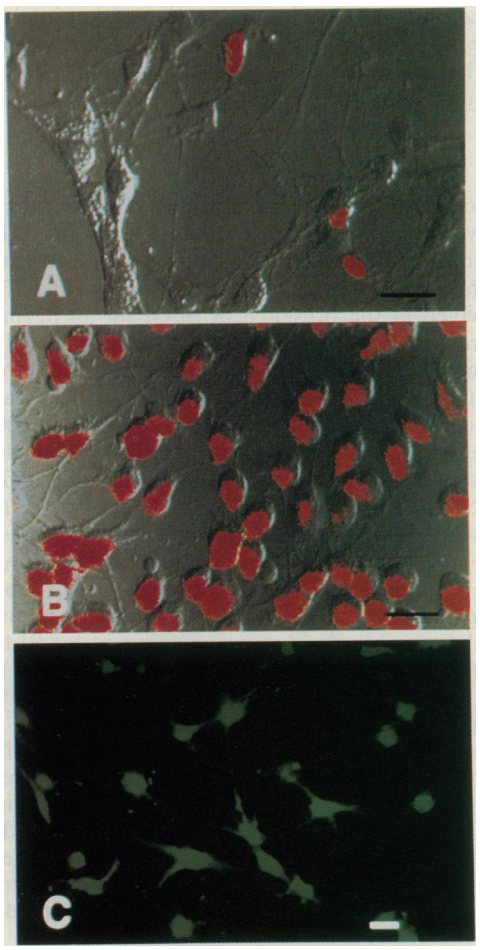

FIG. 2. BrdUrd staining and NeuroTag binding of neurons in culture. Neurons were labeled with BrdUrd for 1 day (*A*) and 4 days (*B*). A small fraction stained on day 1, but by day 4, all cells were stained with BrdUrd. Cell bodies and processes of all cells in culture were stained with a neuronal marker, NeuroTag (*C*). (Bar = 20 μm.)

processes than cells newly cultured from the brain (Fig. 3*B*), suggesting that many processes survived passaging. Cells passaged and kept in culture for 14 days in the presence of bFGF formed aggregates interconnected by an extensive network of processes forming a lattice-type pattern (Fig. 3*C*). Few cells divided in open areas; most cell division occurred in the aggregates.

The cultures were characterized by immunostaining for various antigenic markers (Fig. 3 *D–F*; Table 1). All cell somata and their processes immunostained strongly with antibodies against NF protein (Fig. 3*D*) and NSE (Fig. 3*E*). NeuroTag green stained cell bodies and processes of all cells in the culture (Fig. 2*C*), indicating that the cells were neurons and that few if any nonneuronal cells were present. The large optical depth of field with the objective used (×10) fails to demonstrate the localization of NeuroTag signal as membrane bound.

The cultures were tested for nonneuronal cells (Table 1). Lack of immunostaining with antibodies against GFAP, galactocerebroside, vimentin, and fibronectin indicated the absence of astrocytes (Fig. 3*F*), oligodendrocytes, and fibroblasts (Table 1). In control experiments, anti-GFAF (Amersham) and anti-vimentin antibodies stained rat C6 and 9L and human U373 glioma cells (data not shown). Table 1 shows the results of immunostaining for all antigenic markers tested, indicating that our neuronal cultures were nearly pure.

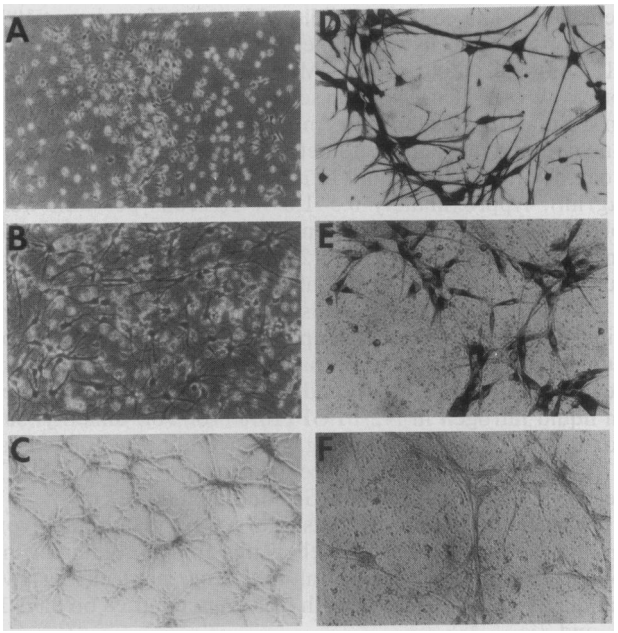

FIG. 3. Cell morphology during culture and passaging (*A–C*) and immunostaining of cells for antigenic markers (*D–F*). (*A*) Primary cell culture after 4 days of plating in N2/bFGF. (*B*) Cells 4 days in culture after passage 3. Compared to *A*, cells are larger and interconnected by processes. (*C*) Cells passaged (passage 3) and kept in culture for 14 days. (*D*) Cells stained with anti-NF antibody. (*E*) Cells stained with anti-NSE antibody. (*F*) Cells stained with anti-GFAP antibody. All cells stained with anti-NF or anti-NSE antibodies, no staining is observed with anti-GFAP antibody. (*A–C*, ×90; *D–F*, ×120.)

Ultrastructural analysis of neurons in culture demonstrated their histotypic neuronal morphology (Figs. 4 and 5), in agreement with previous studies (22–24). The well-differentiated neurons exhibited a histotypic pyramidal morphology, including a primary apical dendrite with multiple ramifications, finer caliber axons, and characteristic nuclear morphology. A TEM micrograph of a pyramidal hippocampal neuron is shown in Fig. 4*A*. The level of this section encompasses both the soma and processes, including a major apical process and a finer-caliber process emerging from the basal aspect of the soma. The nucleus has peripheral heterochromatin and a reticulated nucleolus (Fig. 4*B*). Mitochondria and microtubules are present in the perikaryal cytoplasm, which is dominated by rosettes of polysomes; a portion of the major apical process contains the same organelles identifying it as a primary dendrite (Fig. 4*C*). Contact between two neuritic processes is shown in Fig. 4*D*. The larger process containing a mitochondrion, microtubules, and vesicles is being contacted by a bouton-like structure containing coated vesicles. Although the membranes at the site of contact appear to be

Table 1. Antigenic properties of primary hippocampal neurons

Antigenic marker	Labeling
NF (neurons)	++
GFAP (glia)	−
Nestin (stem cells)	++
Vimentin (glia precursors/fibroblasts)	−
NSE (neurons)	+
OX-42 (microglia/macrophages)	−
Galactocerebroside (oligodendrocytes)	+
Microtubule-associated protein 2 (dendrites)	+
bFGF receptor (neurons/glia)	+
Fibronectin (fibroblasts)	−

++, Strongly labeled; −, not labeled; +, weakly labeled.

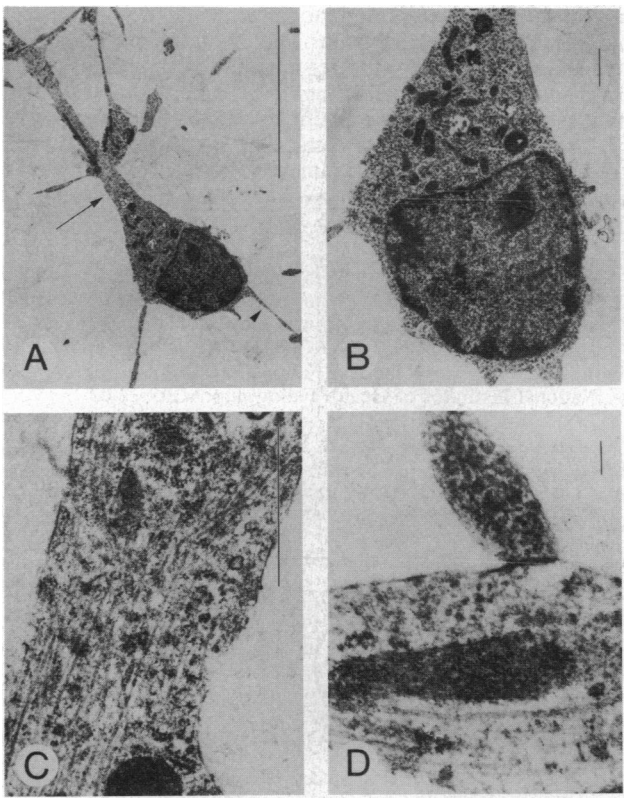

FIG. 4. TEM micrographs of cultured neurons. (*A*) A pyramidal neuron including a major apical process (arrow) and a finer caliber process (arrowhead). (Bar = 10 μm.) (*B*) Enlargement of neuronal soma in *A*. (Bar = 1 μm.) (*C*) A portion of the major apical process shown in *A*. This process is dominated by microtubules and polysomal ribosomes identifying it as a primary dendrite. (Bar = 1 μm.) (*D*) Contact between two neuritic processes. (Bar = 0.1 μm.)

uniformly parallel, there is little indication of further assembly of synaptic structures.

SEM showed some well-differentiated pyramidal somata (Fig. 5*A*) extending large processes with multiple levels of branching and some less-differentiated rounded neurons extending long processes. Major dendritic processes arising from the well-differentiated neurons are large caliber processes with acute bifurcations (Fig. 5*B*) and small-caliber axon-like branches. Well-differentiated neurons typically possess a large pyramidal soma (Fig. 5*C*; compare to Fig. 4*A*). Some less-differentiated neurons were fixed in the process of dividing (Fig. 5*C*). Although cytokinesis is apparently underway, the membrane connecting the two daughter cell components is clearly continuous (Fig. 5*D*). The daughter cell component to the right (Fig. 5*D*) is extending a fine-caliber, possibly axonal, process into the foreground. Extending from this component into the upper right of the field is another thicker dendrite-like process with several levels of branching.

In contrast to the previous ultrastructural reports (20, 21, 23), fine-caliber axonal processes emerged from the soma in a histotypic manner in addition to the dendritic origin previously described (Figs. 4*A* and 5*D*). These somatic axonal extensions may result from high levels of trophic support. Less-differentiated neurons typically had rounder somata with fewer less-elaborate processes. Rounded neurons, differentiated adequately to extend processes, appeared capable of proliferating (Fig. 5*D*). We have been able to identify neuronal processes and somata with confidence based on both the ultrastructural surface morphology and organelle content that clearly demonstrate that both well-differentiated and proliferating less-differentiated cells are neurons.

DISCUSSION

Our results demonstrate that bFGF has both survival and proliferative effects on hippocampal neurons and exhibits a distinct dose–response pattern. Lower concentrations promote survival as reported (4–8, 19), and higher concentrations induce long-term cell proliferation. Although the practical significance of this work lies in our success in establishing long-term neuronal cultures, the demonstration here of *in vitro* proliferation and the observed dose–response effect raise questions concerning the role of bFGF in survival and potential for growth of neurons *in vivo*.

The differential effect of low and high bFGF concentrations suggests the involvement of high- and low-affinity receptors in survival and proliferation, respectively. Both types of receptors are present in all parts of the central nervous system (25, 26) and the expression of high-affinity receptors is temporally regulated (27). If the *in vitro* dose–response effect has physiological significance *in vivo*, bFGF

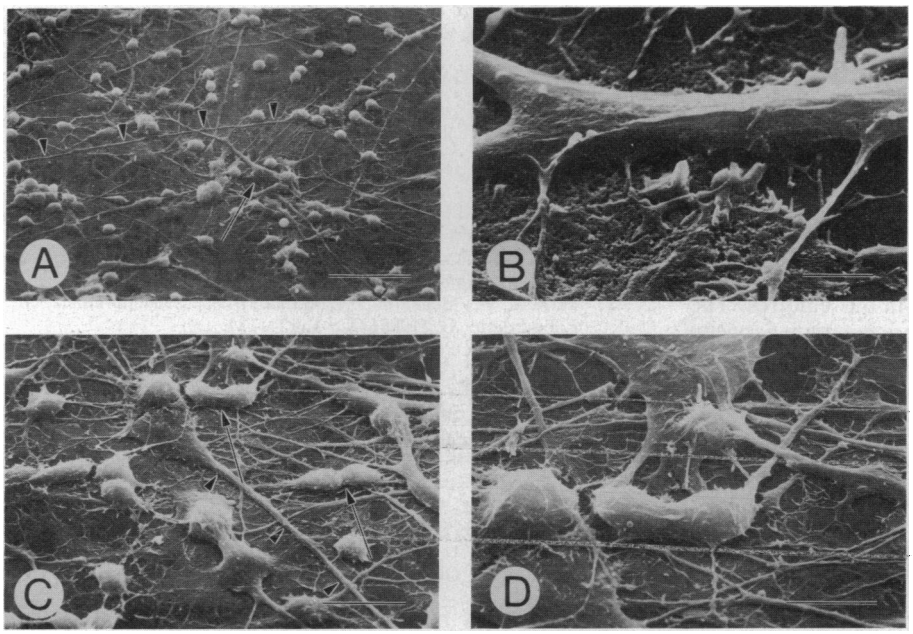

FIG. 5. SEM micrographs of hippocampal neurons in culture. (*A*) Neurons in culture including well-differentiated pyramidal somata (arrow) and less-differentiated rounded neurons that extend processes (arrowheads). (Bar = 50 μm.) (*B*) A dendrite from a well-differentiated neuron showing a major bifurcation and smaller collaterals. (Bar = 2 μm.) (*C*) A well-differentiated neuron with a large apical dendrite (arrowheads) is contacted by a number of neuronal processes from other neurons; other less-differentiated neurons are seen in the process of dividing (arrows). (Bar = 20 μm.) (*D*) Enlarged view of a process-bearing dividing neuron where the membrane is clearly continuous. (Bar = 10 μm.)

may have temporal and/or spatial gradients *in vivo*. In the simplest scenario, the rat hippocampal neurons may not proliferate *in vivo* because they do not normally experience high bFGF concentrations. The concentration experienced by the receptors in their microenvironments may be low because most bFGF is bound to the cell surface (28) and is, therefore, inaccessible. Under certain conditions (e.g., cell death or injury), bFGF released from cells may become available in a dose–response manner, depending on the position of a particular cell in relation to the cells releasing bFGF. We recognize, however, that *in vivo* regulation of cell survival and proliferation may be more complex, possibly involving additive or subtractive effects of other growth factors, such as nerve growth factor, and other modulators.

bFGF has mitogenic effects on astrocytes and oligodendrocytes (29, 30). Further, astrocytes enhance neuronal survival (31). It was therefore important to ascertain that the trophic, tropic, and proliferative effects in our cultures were not due to the presence of astrocytes. Short-term virtually pure neuronal cultures have been grown in the chemically defined medium N2 (17, 18, 32–34), which does not support the survival or proliferation of nonneuronal cells, making it possible to obtain >95% pure neuronal culture. Our cultures may also have been uncontaminated by glia because cells from E18 fetal hippocampi are homogeneous, consisting mainly of pyramidal neurons (20, 21). We characterized the neuronal nature of the cultures and established the absence of nonneuronal contaminants through morphological, ultrastructural, and immunocytochemical analyses.

Cultures were characterized by immunostaining for several antigenic markers, TEM and SEM. The cultures were immunostained with antibodies against NF protein (Fig. 2*A*) and NSE (Fig. 2*E*), indicating their neuronal nature, which is consistent with previous reports that cultured hippocampal (E16) and septal (E18) neurons stain with anti-NF antibody (35, 36). Proliferating neuroepithelial cells from mesencephalon and telencephalon in culture expressed NF in the presence of higher concentrations of bFGF (>0.5 ng/ml) (10). Spinal cord neurons cultured in the presence of bFGF (5 ng/ml) expressed NSE (11). In the present study, primary hippocampal cells also expressed other neuron-specific markers such as nestin, an intermediate filament protein, and microtubule-associated protein 2 (Table 1).

Electron microscopy showed that the cultured cells had typical neuronal morphologies. Although cells of diverse morphologies were seen by SEM, some well-differentiated pyramidal cells with extended processes were present in the cultures. In the TEM, cell morphology was similar to that in previous studies of cultured hippocampal neurons (35). However, contrary to previous reports, besides the dendritic fibers, fine axonal processes also emerged from the soma (Figs. 3*A* and 4*D*). The proliferation of differentiated process-bearing neurons may indicate that high levels of bFGF simultaneously exert powerful proliferative and differentiation effects on hippocampal neurons.

Mixed population of cells derived from rat and mouse brains can become transformed (15, 16). We have not observed any evidence of transformation in our cultures. The cellular morphology and growth rates were invariant between passages. Moreover, when these cells were implanted into the brain of adult syngeneic animals, they did not form tumors (unpublished data).

In addition to pointing out the significance of trophic factors for survival, growth and proliferation, the present study underscores the importance of considering qualitative variability in cellular response to different concentrations of a single growth factor *in vivo*. Our ability to maintain primary hippocampal neurons in long-term culture suggests that, by using specific combinations of trophic factors, it may be possible to generate continuous neuronal cell lines from different regions of the brain. The development of primary hippocampal neuronal cultures maintained as cell lines, without oncogenic immortalization, should permit investigation of questions regarding the biochemical and cellular properties of these cells and the dynamics of interaction with their cellular and chemical environment. These cells could also be genetically manipulated *in vitro* to express specific genes for use in somatic gene transfer.

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